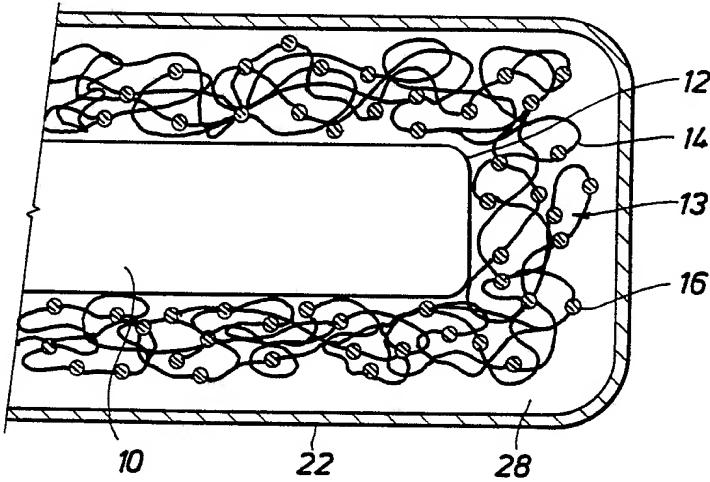


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(54) Title: IMPROVED ENZYME ELECTRODES**(57) Abstract**

An improved enzyme electrode (10) includes a three-dimensional redox polymer network (14) having redox enzymes (16) bound thereto, and also having a protein excluding polymeric coating (22).

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TITLE: IMPROVED ENZYME ELECTRODES

This is a continuation-in-part of co-pending application U.S. Serial No. 389,226, filed August 2, 1989.

5

FIELD OF THE INVENTION

This invention relates to amperometric biosensing electrodes for the detection of a specific analyte in a test sample. More 10 particularly, this invention relates to novel biosensors containing a polymeric overcoating which confers increased sensitivity to the electrodes when used in biological fluids.

15 BACKGROUND OF THE INVENTION

Amperometric biosensors are capable of quantifying trace amounts of biological analytes such as glucose, urea, cholesterol, and the like in biological fluids. Analyte may be electroxidized 20 directly at the electrode, or an enzyme may be immobilized on an electrode such that the reaction product of the enzyme with its substrate is detected by an electrical change, e.g., change in current flow, at the electrode. The current generated at 25 the electrode is a function of the quantity of analyte in a sample.

Historically, electrical communication between enzyme and electrode has been achieved through the use of diffusing mediators. These include small

diffusing redox shuttles such as ferrocenes, quinones, ruthenium amines, and the like. Enzyme electrodes using such mediators generally require that the enzyme and mediator shuttle be confined to 5 the proximity of the electrode surface. The small shuttle molecules commonly employed can, however, readily diffuse through membranes that contain the enzyme. These membranes must allow passage of the enzyme's substrate, e.g., glucose and product e.g., 10 gluconate or gluconolactone. Diffusion of the mediators away from the electrode surface results in reduced sensitivity of the electrode.

The inventors have previously reported the production of novel amperometric biosensors having a 15 three-dimensional redox polymer network to which a redox enzyme is chemically bound. The three-dimensional redox molecular structure provides electrical contact between the surface of the electrode and the redox enzyme. Such electrodes are 20 described in co-pending application U.S. Serial No. 389,226 which is hereby incorporated by reference as if fully set forth and in Gregg and Heller, J. Phys. Chem., 95:5970-5980 (1990) and Heller, Accounts of Chem. Res., 23:128 (1990).

25 The three-dimensional redox network of these electrodes is preferably formed of a redox enzyme, a cross-linking agent, and a cross-linkable compound capable of reacting with the cross-linking agent and the redox enzyme. Either the cross-linkable 30 compound or the cross-linking agent, or both, have multiple redox centers. When the compounds of each

embodiment are mixed together under appropriate conditions, a chemical reaction takes place resulting in the formation of a cross-linked, three dimensional, redox polymer with the redox enzymes 5 chemically bound within the cross-linked redox polymer network.

Materials known to produce the three-dimensional redox polymer network and effect the directly wired enzyme electrodes described above are 10 limited. It would be desirable to have provided a variety of materials from which such electrodes could be produced and/or improved.

It has recently been found that the sensitivity of the three-dimensional redox polymer network 15 electrodes described above in biological fluids such as serum or blood is decreased by a factor of 10-100 within 10 seconds as compared with sensitivity in a buffer solution. After loss of sensitivity in such a biological sample, the original sensitivity is 20 recovered if the electrode is rinsed and retested in buffer.

It would be highly desirable to prevent this reversible loss of sensitivity of the three-dimensional redox polymer network enzyme electrodes 25 to obtain an improved enzyme electrodes having high sensitivity.

SUMMARY OF THE INVENTION

The present invention includes novel redox 30 polymers for the production of three-dimensional redox polymer networks for use in amperometric

5 biosensors. The present invention also describes prevention of the reversible loss of sensitivity of three-dimensional redox polymer network enzyme electrodes in biological fluid such as blood and serum by the addition of a barrier to protein diffusion into the electrode, e.g., a thin polymeric film. The polymeric film prevents in-diffusion of proteins, particularly small proteins like albumin, yet allows continued good in-diffusion of redox 10 enzyme substrate and good out-diffusion of reaction products, thereby preventing reduction in current output associated with diffusion of protein in the sample (e.g., albumin) to the 3-dimensional redox network.

15

DESCRIPTION OF THE FIGURES

Fig. 1 is a schematic diagram of an electrode of the present invention.

20 Fig. 2 is a diagrammatic representation of the structure of Polymer I (PVP-Os-NH₂).

Fig. 3 is a diagrammatic representation of the structure of Polymer II (PVI-Os).

Fig. 4 is a diagrammatic representation of the structure of Polymer III (PVI-Os-NH₂).

25 Fig. 5 is a diagrammatic representation of the coated enzyme electrodes of the present invention.

Fig. 6 is a graphical representation of current generated in response to glycollate by a glycollate electrode formed with a PAZ triaziridine-type 30 crosslinker.

Fig. 7 is a graphical representation of current generated in response to cholesterol by a cholesterol oxidase electrode of the present invention.

5 Fig. 8 is a graphical depiction of the current generated in response to glucose by a glucose electrode containing PVI-Os redox polymer.

10 Fig. 9 is a graphical depiction of the current generated in response to glucose by a glucose enzyme electrode containing a PVI-Os-NH₂ redox polymer.

Fig. 10 is a graphical depiction of the current generated in response to glucose in buffer or serum by a glucose electrode (non-coated).

15 Fig. 11 is a graphical depiction of the current generated in response to glucose in buffer or serum by a glucose electrode which has been coated with polyurethane.

20 Fig. 12 is a graphical depiction of the current generated in response to glucose in buffer or serum by a glucose electrode which has been coated with Nafion.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

25 As shown in Fig. 1, a biosensor of the present invention includes an electrode 10 having a test surface 12. The surface 12 is substantially covered with a three-dimensional redox polymer 14 to which is bound a redox enzyme 16. The three-dimensional redox polymer network 13 thus formed electrically connects the electrode 10 to a redox enzyme 16.

The electrode 10 may be formed of any material known for the manufacture of biosensing electrodes. Preferably the electrode is formed of a solid material, e.g., glassy carbon. Additional suitable 5 electrode materials include graphite, platinum, palladium, tin oxide, and conducting organic salts.

The three-dimensional redox polymer includes at least two components which can combine to form a 10 three-dimensional molecular structure. At least one of these components comprises a redox compound, and at least one other component comprises a redox enzyme. The resulting three-dimensional molecular structure has multiple redox centers and has the 15 redox enzyme chemically bound within.

The term "bound within" describes a redox enzyme which is chemically, and preferably covalently, bonded to the redox polymer.

The redox enzyme may be glucose oxidase, D- or 20 L-amino acid oxidase, lactate oxidase, and the like. It has now been found that cholesterol oxidase and theophylline oxidase are useful enzymes in the present invention.

The term "redox compound" is used herein to 25 mean a compound that can be oxidized and reduced. The redox compound may have one or more functions that are reducible and oxidizable. Further, the term "redox compound" means a compound which contains one or more redox centers, "redox center" 30 meaning a chemical function that accepts and transfers electrons.

The redox compounds, or redox centers contained within compounds useful in this invention may be organic or inorganic. Transition metal complexes with organic ligands such as bipyridine are 5 preferred as redox centers because of their chemical stability and various oxidation states and their fast electron transfer kinetics. Examples of such complexes include polypyridine complexes of di- or trivalent osmium ions. Organic redox centers may 10 also be employed, e.g., various derivatives of viologen (*N,N'*-bis alkyl-4,4'-bipyridine). A number of preferred crosslinkable compounds containing redox active centers are known. Some of these compounds require only the addition of enzymes to 15 form 3-dimensional crosslinked films, i.e., the enzyme is the only required crosslinking agents. Other compounds do not directly react with chemical functions present on the enzyme and thus require a separate crosslinking agent to form the 3- 20 dimensional network.

A preferred redox polymer complex for use in the present invention is Polymer I, poly(vinylpyridine) complexed with [osmium bis (2,2'-bipyridine)Cl] (PVP-Os) which may be 25 quaternized, e.g., with bromoethylamine to form a highly hydrophilic crosslinkable redox polymer containing pendant ethylamine groups (PVP-Os-NH₂) as shown in Fig. 2. Novel redox polymers useful in this invention also include derivatives of poly(*N*-vinyl imidazole) (PVI) complexed with [Os(bpy)₂Cl]⁺²⁺ 30 (PVI-Os) (Polymer II), shown, for example, in Figure

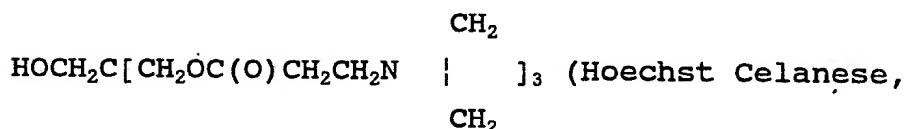
3. PVI-Os is a crosslinkable redox polymer, but may also be quaternized to form PVI-Os-NH₂ (Polymer III, shown in Figure 4) for additional crosslinking sites.

5 In a preferred embodiment, the three-dimensional redox polymer network includes a redox enzyme, a cross-linking agent, and a cross-linkable compound capable of reacting with the cross-linking agent and the redox enzyme. Either the cross-
10 linkable compound or the cross-linking agent, or both, contain multiple redox centers.

Preferred cross-linking agents are water soluble compounds that react under conditions where most enzymes are stable, that is, in water, between 15 pH3 and pH9 at 0°-50°C. Included in this category of cross-linking agents are multi-functional epoxides such as polyethylene glycol diglycidyl ether (PEGDGE), carbodiimides, di- and poly- aldehydes, imidoesters, and N-hydroxysuccinimid esters. A 20 number of reagents with limited solubility in water may also be used by dissolving them in a water-miscible organic solvent such as acetone, methanol, acetonitrile or dimethylformamide. Included in this category are reagents such as cyanuric chloride, 25 tetrachlorobenzoquinone, and tetracyanoquinodimethane. These reagents may react with one or more types of functions including amines, alcohols, thiols and carboxylic acids which may be present on the surface of enzymes and which 30 may also be included in the structure of the redox compound.

Additional crosslinkers which have now been found to be useful in the preparation of biosensors include di-, tri-, and poly-aziridines, e.g., pentaerythritol-tris-(B-(N-aziridinyl) propionate,

5



Charlotte, N.C.) and the like.

Preferred crosslinkable compounds are 10 hydrophilic, containing chemical groups such as alcohols, carboxylic acids, amines, sulfonates, sulfates, phosphates, and phosphonates. Such groups tend to promote the solubility of the components in water which facilitates contact with the water 15 soluble enzymes. Such groups may also improve the stability of the immobilized enzyme against denaturation.

To form the inventive electrodes, the 20 components of the three-dimensional redox polymer network are mixed together under appropriate conditions such that a chemical reaction takes place resulting in the formation of a three-dimensional redox polymer having redox enzyme bound within a three-dimensional redox polymer network.

25 Mixture of redox enzyme and the various polymer components in a common solution is followed by the application of the solution to an electrode surface. Various application methods may be used, including the addition of drops of the solution onto the 30 electrode surface, dip coating, spin coating, or spraying the solution onto the electrode surface.

The application step is followed by a curing step such as drying in air or vacuum. Alternatively, the process may involve the addition of the enzyme and polymer components in separate solutions to the 5 surface of the electrode, mixing, and then curing in air in an inert atmosphere or under vacuum.

In a preferred embodiment, as shown in Fig. 5, the improved enzyme electrode of the present invention also includes a polymeric film 22 which 10 prevents in-diffusion of macromolecules, generally of molecular weights greater than 5000, such as serum albumin, from the assayed solution. The polymeric film 22 is preferably as thin as is practically feasible, subject to its having a low 15 density of holes. The polymeric film 22 must be permeable to the substrate of redox enzyme 16 to allow good substrate in-diffusion and also permit good out-diffusion of product of the redox enzyme catalyzed reaction, i.e., gluconolactone or 20 gluconate for a glucose electrode.

The polymeric film 22 may be directly applied to the surface of the redox polymer network 13, e.g., in direct physical contact, or may be positioned at a distance from the surface of the 25 network 13, creating a space 28 between the film and the network 13. The latter may be preferred, for example, where the electrode 10 is located within a probe, e.g., a trochanter, into which sample solution may enter through a relatively narrow port. 30 In this case, the polymeric film may span the entry

port and thereby exclude communication of proteins with the redox polymer network.

It is hypothesized that protein such as albumin present in a biological sample may compete for redox polymer segments of the three-dimensional redox network and competitively replace segments of redox enzyme in the enzyme polymer complexes, may adversely interact with the enzyme, or both. The enzyme may remain covalently bound to the network even if some of the redox-network complex segments are competitively displaced by competing protein. Such complexing may reduce the fraction of electrons transported from the enzyme to the redox polymer network and thereby reduce the current generated at a given substrate concentration.

A polymeric film, through which the redox enzyme substrate and its generated products freely diffuse, but competing proteins such as albumin do not diffuse will be useful in the present invention. The preferred films are polyanionic, i.e. have at pH7 multiple bound anions, as polyanionic membranes exclude electrooxidizable anionic interferants. The inventive membranes exclude proteins and preferably exclude both proteins and electrooxidizable anions like urate and ascorbate. Examples of useful films include polycarbonate and polyurethane films. Especially preferred is Nafion, a commercially available, chemically-inert, polyanionic hydrophilic polymer. Most preferably, an electrode of the present invention effectively prevents undesired protein interactions with the enzyme/redox polymer

layer, and also effectively eliminates small molecule interferants, as discussed in co-pending patent application U.S. Serial No. 753,812 filed September 3, 1991, which is hereby incorporated by reference as if fully set forth. The inclusion of a peroxidase or peroxidase-like catalyst reduces the number of interfering substances recognized at the electrode. Alternatively, anionic sites provided in the preferred protective films such as Nafion or 5 palmitic acid-containing polyurethane films effectively exclude anionic interferants while the films prevent protein interactions with the redox polymer network. 10

Anionic sites may be included in the polymeric protective films. For example, multiple anionic functions may be provided in films containing carboxylate, sulfonate, phosphate, or phosphonate 15 functions covalently bound to the polymer. In a preferred embodiment, the polymeric film contains 20 fluorine.

In the method of the present invention, the inventive enzyme electrode is immersed into a test solution containing analyte, e.g., glucose. The analyte diffuses into the 3-dimensional redox 25 polymer network where it may react with the redox enzyme, e.g., glucose oxidase, forming the reduced form of the enzyme and oxidized form of the analyte (e.g., gluconolactone). The reduced redox enzyme may then be oxidized by the redox centers of the 30 redox polymer, e.g., the osmium complex. Electrons are subsequently transferred through the polymer to

the electrode and the electrical current increases linearly with the concentration of the substrate over a substrate concentration range.

In the preferred, polymer-coated enzyme electrode of the present invention, the analyte (e.g., glucose) must readily diffuse through the polymeric coating to reach the redox enzyme. Also, the reaction product generated (e.g., gluconolactone or gluconate) must freely diffuse through the polymeric coating for quick removal. Interfering proteins present in biological fluids, e.g., albumin are prevented from contacting the redox polymer by the protective polymeric coating. In the most preferred embodiment, the polymer-coated enzyme electrode also contains a means to exclude anionic interferants, e.g., as discussed above.

EXAMPLES

The following examples are designed to illustrate certain aspects of the present invention. The examples are not intended to be comprehensive of all features and all embodiments of the present invention, and should not be construed as limiting the claims presented herein.

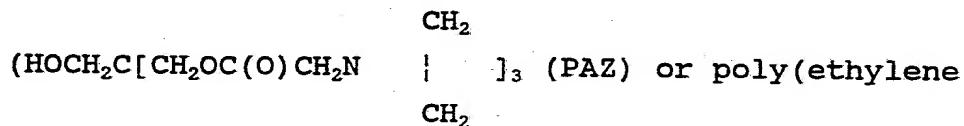
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EXAMPLE 1

Aziridine Crosslinker

Electrodes where one enzyme of the group D-amino acid oxidase, L-amino acid oxidase, glycollate oxidase, cholesterol oxidase, theophylline oxidase, sarcosine oxidase, or galactose oxidase, was

electrically connected through a 3-dimensional redox polymer network to an electrode were prepared as follows: First, poly(vinyl pyridine) was complexed with $[\text{Os}(\text{bpy})_2 \text{Cl}]^{+/-2+}$ and then partly quaternized 5 with ethylamine as described in copending U.S. Patent application serial No. 389,226, to form Polymer I, PVP-Os-NH₂ as shown in Figure 2. The resulting macromolecule was then complexed with one of the enzymes. The enzyme-polymer complexes were 10 next crosslinked using hydroxylated triaziridine



glycol) diglycidyl ether (PEGDGE). The ratio of 15 enzyme to polymer was 0.57 ± 0.1 by weight. The ratio was adjusted to keep the total enzymatic activity on each of the different enzyme electrodes at approximately 0.01 units/electrode. The cross-linker containing enzyme-polymer complex solution 20 was next deposited dropwise onto a polished vitreous carbon disk electrode. The electrode was cured for approximately 24 hours in a vacuum desiccator at 22°C. The cured electrode was then extensively washed in pH 7.2 phosphate buffer, while rotated at 25 1000 rpm, for 3-5 hours. The thickness of the resulting films (when wet) was 1-2 μm .

The prepared electrodes were used in a test solution of 30mM phosphate buffer (pH 7.2) with 0.15M NaCl, except in the case of cholesterol 30 oxidase, where this buffer was diluted with an equal volume of isopropanol and contained 0.5% Triton X-

100. The electrodes were poised at 0.45V (SCE). After a steady base line was reached a substrate solution was injected. The substrates included D-alanine for D-amino acid oxidase; L-leucine for L-amino acid oxidase; glucose for glucose oxidase; cholesterol for cholesterol oxidase, etc. In the case of theophylline oxidase, theophylline addition made the test solution more acidic. The electrodes were rotated during the assay at 1000 rpm.

10 Table I lists the characteristics of some of these prepared electrodes when each was used to measure its specific substrate, including the current density, apparent Km, and sensitivity. Each data point is the mean of four electrode

15 measurements.

TABLE I

Enzyme	Crosslinker	Electrode Km (mM)	Highest Current Density (μAcm^{-2})	Sensitivity* ($\text{AM}^{-1}\text{cm}^{-2}$)
D-AMINO ACID OXIDASE	PAZ	1.000	20.0	0.0100
20 THEOPHYLLINE OXIDASE	PAZ	0.047	2.9	0.0310
GLYCOLLATE OXIDASE	PEGDGE	0.900	17.0	0.0090
25 CHOLESTEROL OXIDASE	PEGDGE	0.250	2.3	0.0048
GALACTOSE OXIDASE	PEGDGE	30.000	100.0	0.0030

* at the substrate concentration corresponding to the apparent Km.

30

The steady state current response of the glycollate oxidase-containing electrode to varying concentrations of glycollate is shown in Figure 6; Figure 7 shows the steady state current response of 5 the cholesterol oxidase-containing electrode when cholesterol (in isopropanol) was injected so as to bring the total cholesterol concentration to the indicated level. e.g., 0.024-0475mM. Figures 6 and 7 show that the currents increase with substrate 10 concentration over the concentration ranges shown.

EXAMPLE 2

Preparation of PVI-Os

Vinyl imidazole was mixed with water at a ratio 15 of 2:1 (by weight) and the resulting 7M solution was purged with argon for 15 minutes. The container was then sealed and δ -irradiated by a radioactive cobalt source (Co-60) with 2.5MRad at a rate of 100kRad/hour. The resulting polymer was then 20 precipitated by adding the irradiated solution to 4L of a mixture of 1:2 acetone:hexane. The precipitated polymer was redissolved in MeOH and again reprecipitated from 1:2 acetone:hexane. The poly(vinyl imidazole) (PVI) was then dried in a 25 vacuum at 90°C.

The PVI (1.92 grams) was next further purified by dissolving in 30mL MeOH and precipitating with ether (2L). This dissolution-precipitation was repeated for a total of three times. The 30 precipitated PVI weighing 380mg, and 456mg of Os(2,2'-bis bipyridine)₂Cl₂ were dissolved in 100ml

5 ETOH. The solution was refluxed under nitrogen for
 five days. The resulting complex, PVI-Os, (Polymer
 II, as shown in Figure 3) was precipitated by adding
 2L ether and the precipitate dried in a vacuum at
 38°C for 12 hours. This procedure follows that
 described by Forster and Vos, Macromolecules,
 23:4372-4377, 1990, except that more of the osmium
 complex was reacted with the PVI and the osmium
 complex content of the PVI-Os was correspondingly
10 higher.

15 Unlike PVP-Os, PVI-Os itself may be cross-
 linked with an enzyme, using a crosslinking agent to
 form a 3-dimensional redox polymer network of the
 inventive electrodes. Alternatively, PVI-Os may be
 quaternized to form PVI-Os-NH₂ (Polymer III of Figure
4) which contains additional crosslinkable
 ethylamine functions. To form PVI-Os-NH₂, 600mg
 PVI-Os was added to 1.23g 2-bromoethylamine
 dissolved in 18ml ethylene glycol and 30ml dimethyl
20 formamide. This mixture was reacted at 64°C for 24
 hours. The resulting polymer was then precipitated
 by adding the solution of PVI-Os-NH₂ to acetone (2L)
 and filtered. The polymer was redissolved in 30ml
 H₂O and the bromide ions were exchanged with chloride
25 using an anion exchange resin. The beads were
 filtered after 24 hours, then 20ml of H₂O was added
 to the filtered solution, which was dialyzed against
 water for 48 hours. A Spectrapor™ dialysis membrane
 having a molecular weight cutoff of 3500 was used
30 for dialysis. The PVI-Os-NH₂ polymer was obtained
 upon vacuum evaporation of the water.

EXAMPLE 3Glucose Electrodes Formed of PVI-Os and PVI-Os-NH₂

Glassy carbon 3mm diameter electrodes were polished with sand paper (400 and 600 grit) and with 5 alumina (20, 5, 1 and 0.3 μ m). A glucose electrode was prepared by the sequential addition of 1 μ l PVI-Os or 1 μ l PVI-Os-NH₂ polymer, 5mg/ml in H₂O; 1 μ l glucose oxidase (GOD), 5mg/ml in H₂O; and 1 μ l PEGDGE, 0.5mg/ml in H₂O, dropwise onto the electrode, with 10 stirring. The electrode was permitted to cure in air at room temperature for 15 hours.

The glucose electrodes made with PVI-Os and PVI-Os-NH₂ polymers were tested in a test solution to which increasing amounts of glucose were added.

15 These glucose calibration curves were obtained under argon at 21.3°C and 1000 rpm electrode rotation. Figure 8 shows the current generated in response to glucose from the glucose oxidase electrode prepared from the novel redox polymer PVI-Os. Figure 9 shows 20 the current generated in response to glucose from the glucose oxidase electrode prepared from the novel redox polymer PVI-Os-NH₂.

EXAMPLE 4

25 Preparation and Testing of 3-Dimensional Redox Polymer Enzyme Electrodes

Three-dimensional redox polymer glucose electrodes

were prepared and tested in the presence of glucose 30 as described previously (see copending patent application U.S. Serial No. 389,226; Gregg and

Heller, J. Phys. Chem., 95:5970-5980, 1991; and Pishko et al., Anal. Chem., 63:2268-2272, 1991). The electrodes were tested in the presence of glucose in phosphate buffer as compared with serum 5 samples. The results as shown in Fig. 10 demonstrate a difference in the response of the glucose sensor in phosphate buffer as compared with serum. A decrease in sensitivity of the glucose electrode was seen in the serum sample as compared 10 with the buffer sample. The loss of sensitivity of these electrodes in the serum sample was substantial.

EXAMPLE 5

15 Glucose Electrode Containing Polyurethane Overcoating

A three-dimensional redox polymer glucose electrode was prepared as described for Example 4. The prepared glucose electrode was then coated with 20 a mixture of medical-grade polyurethane (PELLETHANE, Dow Chemical) and palmitic acid. The polyurethane served as a protein exclusion barrier and palmitic acid served to place negative charges within the polyurethane film to exclude ascorbate and urate. 25 The polymeric film was applied to the electrodes' surface by dip coating the electrode in 2% polyurethane, 1% palmitic acid codissolved in tetrahydrofuran (THF). The polymeric film was allowed to solidify on the electrodes in humidified 30 air for six hours, after which the film-coated electrodes were washed in phosphate buffered saline

for two days so as to hydrate the polymeric film and remove any residual solvent.

The coated enzyme electrodes were then tested in glucose containing solutions prepared in either 5 phosphate buffered saline (buffer) or in serum. The results, shown in Fig. 11, demonstrated no loss of sensitivity of the coated glucose electrode in the serum sample. The difference in glucose sensitivity was small (approximately 15%). The palmitate anions 10 in the film were effective in excluding interfering ascorbate and urate anions from the steep increase in current seen in the absence of an interference eliminating layer (J. Am. Chem. Soc., 113:9003-4, 1991). There was no such increase when 15 physiological concentrations of urate or ascorbate were added.

EXAMPLE 6

Glucose Electrode Containing NAFION Overcoating

20 A three-dimensional redox polymer glucose electrode prepared as described for Example 4 was coated with a perfluorosulfonate ion exchange resin (NAFION™, purchased from Aldrich Chemical Company). Nafion was applied to the glucose electrode surface 25 by dipping the electrode into a solution of 0.5% Nafion in 90% ethanol, 10% H₂O. The ensuing droplet on the electrode surface was allowed to dry in air for approximately two hours. The electrode was then dip coated again, dried, and then soaked in 30 phosphate buffered saline for two hours prior to use. The glucose concentration dependence of the

current of the Nafion-coated electrode in serum as compared with buffer (PBS) is shown in Fig. 12. Only a minor difference between the two response curves was observed.

5

WE CLAIM:

1 1. An enzyme electrode comprising:
2 an electrode having a testing surface; and

3 a transducing film substantially covering
4 the testing surface of the electrode which film is
5 formed by curing on the electrode surface a solution
6 comprising a redox polymer and a hydroxylated di-,
7 tri- or poly- triaziridine as a crosslinking agent.

1 2. The electrode of claim 1, wherein the
2 transducing film comprises a three-dimensional redox
3 polymer network to which a redox enzyme is
4 chemically bound.

1 3. The electrode of claim 2, wherein the
2 hydroxylated di- or poly- triaziridine is
3 pentaerythritol-tris-(B-(N-aziridyl) propionate).

1 4. An enzyme electrode comprising:
2 an electrode having a testing surface; and
3 a transducing film substantially covering
4 the testing surface which film comprises a three-
5 dimensional redox polymer network to which a redox
6 enzyme is chemically bound, wherein the three-
7 dimensional redox polymer network is formed by
8 curing on the electrode surface a solution
9 comprising a redox polymer derived of poly(vinyl
10 imidazole), and a redox enzyme.

1 5. The enzyme electrode of claim 4, wherein
2 the redox polymer is poly(vinyl imidazole) complexed
3 with di- or tri-valent osmium ions.

1 6. The enzyme electrode of claim 5, wherein
2 the redox polymer is poly(N-vinyl imidazole),
3 complexed with [osmium bis (2,2'-bipyridine)Cl]^{+/+2}.

1 7. The enzyme electrode of claim 5, wherein
2 the curing solution further comprises a di-, tri-,
3 or poly-aziridine crosslinker.

1 8. An enzyme electrode comprising:
2 an electrode having a testing surface; and

3 a transducing film substantially covering
4 the testing surface which film comprises a three-
5 dimensional redox polymer network to which a redox
6 enzyme is chemically bound, wherein the redox enzyme
7 is cholesterol oxidase.

1 9. An enzyme electrode comprising:
2 an electrode having a testing surface; and

3 a transducing film substantially covering
4 the testing surface which film comprises a three-
5 dimensional redox polymer network to which a redox
6 enzyme is chemically bound, wherein the redox enzyme
7 is theophylline oxidase.

10. An enzyme electrode comprising:

2 an electrode having a testing surface;
3 a transducing film substantially covering
4 the testing surface, which film comprises a three-
5 dimensional redox polymer network to which a redox
6 enzyme is chemically bound, wherein the redox enzyme
7 electrically communicates through the film with the
8 electrode thereby transducing substrate flux to an
9 electrical current; and
10 a polymeric film excluding from the
11 transducing film proteins of molecular weights
12 exceeding 5000 daltons.

1 11. The enzyme electrode of claim 10, wherein
2 the polymeric film is polyurethane.

1 12. The enzyme electrode of claim 11, wherein
2 the polymeric film further includes palmitic acid.

1 13. The enzyme electrode of claim 10, wherein
2 the polymeric film is polycarbonate.

1 14. The enzyme electrode of claim 10, wherein
2 the polymeric film includes multiple bound anionic
3 functions.

1 15. The enzyme electrode of claim 14, wherein
2 the polymeric film includes multiple carboxylate,
3 sulfonate, phosphate, or phosphonate functions
4 covalently bound to the polymeric film.

1 16. The enzyme electrode of claim 15, wherein
2 the polymeric film contains fluorine.

AMENDED CLAIMS

[received by the International Bureau
on 19 July 1993 (19.07.93);
original claims 2, 4 and 8-10 amended;
remaining claims unchanged (3 pages)]

1. An enzyme electrode comprising:
an electrode having a testing surface; and
a transducing film substantially covering
the testing surface of the electrode which film is
formed by curing on the electrode surface a solution
comprising a redox polymer and a hydroxylated di-,
tri- or poly- triaziridine as a crosslinking agent.
1. 2. The electrode of claim 1, wherein the
transducing film comprises a cross-linked redox
polymer network to which a redox enzyme is chemically
bound.
1. 2. 3. The electrode of claim 2, wherein the
hydroxylated di- or poly- triarizine is
pentaerythritol-tris-(B-(N-aziridyl) propionate).
1. 2. 3. 4. An enzyme electrode comprising:
an electrode having a testing surface; and
a transducing film substantially covering
the testing surface which film comprises a cross-
linked redox polymer network to which a redox enzyme
is chemically bound, wherein the cross-linked redox
polymer network is formed by curing on the electrode
surface a solution comprising a redox polymer derived
of poly(vinyl imidazole), and a redox enzyme.

1 5. The enzyme electrode of claim 4, wherein the
2 redox polymer is poly(vinyl imidazole) complexed with
3 di- or tri-valent osmium ions.

1 6. The enzyme electrode of claim 5, wherein the
2 redox polymer is poly(N-vinyl imidazole) complexed
3 with [osmium bis (2,2'-bipyridine)Cl]^{+/+2}.

1 7. The enzyme electrode of claim 5, wherein the
2 curing solution further comprises a di-, tri-, or
3 poly-aziridine crosslinker.

4

1 8. An enzyme electrode comprising:
2 an electrode having a testing surface; and
3 a transducing film substantially covering
4 the testing surface which film comprises a cross-
5 linked redox polymer network to which a redox enzyme
6 is chemically bound, wherein the redox enzyme is
7 cholesterol oxidase.

1 9. An enzyme electrode comprising:
2 an electrode having a testing surface; and
3 a transducing film substantially covering
4 the testing surface which film comprises a cross-
5 linked redox polymer network to which a redox enzyme
6 is chemically bound, wherein the redox enzyme is
7 theophylline oxidase.

1 10. An enzyme electrode comprising:

2 an electrode having a testing surface;
3 a transducing film substantially covering
4 the testing surface, which film comprises a cross-
5 linked redox polymer network to which a redox enzyme
6 is chemically bound, wherein the redox enzyme
7 electrically communicates through the film with the
8 electrode thereby transducing substrate flux to an
9 electrical current; and

10 a polymeric film substantially covering the
11 transducing film and excluding from the transducing
12 film proteins of molecular weights exceeding 5000
13 daltons.

1 11. The enzyme electrode of claim 10, wherein
2 the polymeric film is polyurethane.

1 12. The enzyme electrode of claim 11, wherein
2 the polymeric film further includes palmitic acid.

1 13. The enzyme electrode of claim 10, wherein
2 the polymeric film is polycarbonate.

1 14. The enzyme electrode of claim 10, wherein
2 the polymeric film includes multiple bound anionic
3 functions.

1 15. The enzyme electrode of claim 14, wherein
2 the polymeric film includes multiple carboxylate,
3 sulfonate, phosphate, or phosphonate functions
4 covalently bound to the polymeric film.

STATEMENT UNDER ARTICLE 19

Claims 2, 4 and 8-10 have been amended to clarify and place in better form. The change involves replacing the term "three-dimensional" with the term "cross-linked."

Support for the changes may be found throughout the specification, and particularly on page 2 at lines 25 and following where the three-dimensional redox networks are described as formed from cross-linking reactions. See also, page 3, line 2 "... a chemical reaction takes place resulting in the formation of a crosslinked, three-dimensional redox polymer...."

Such amendments will have no effect of the description or the drawings.

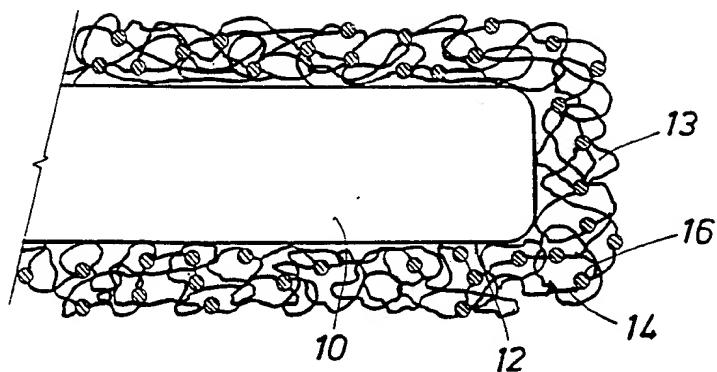


FIG. 1

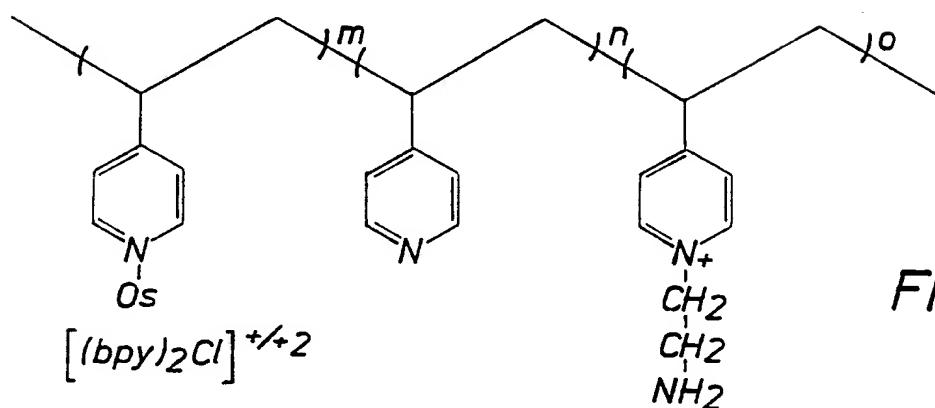


FIG. 2

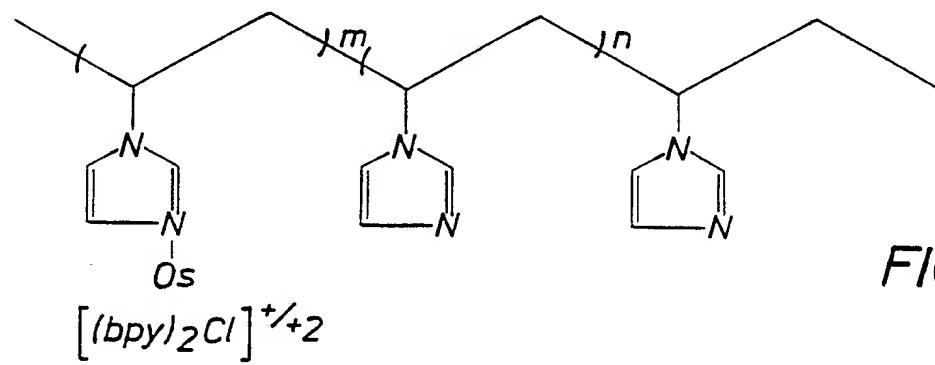


FIG. 3

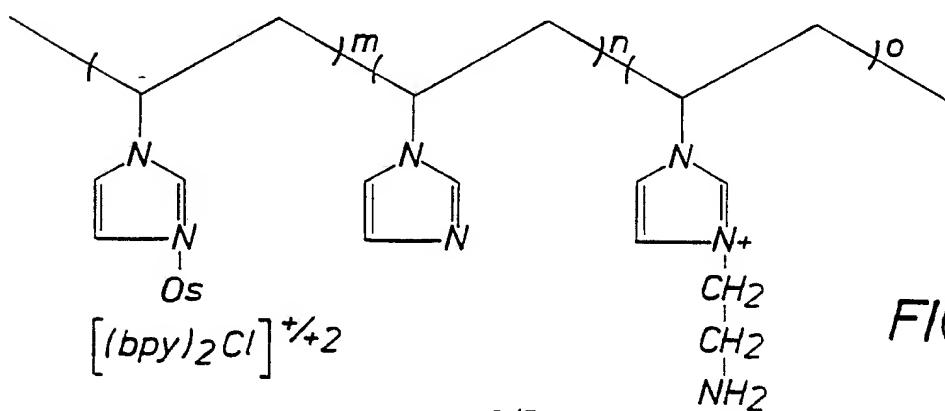


FIG. 4

FIG. 5

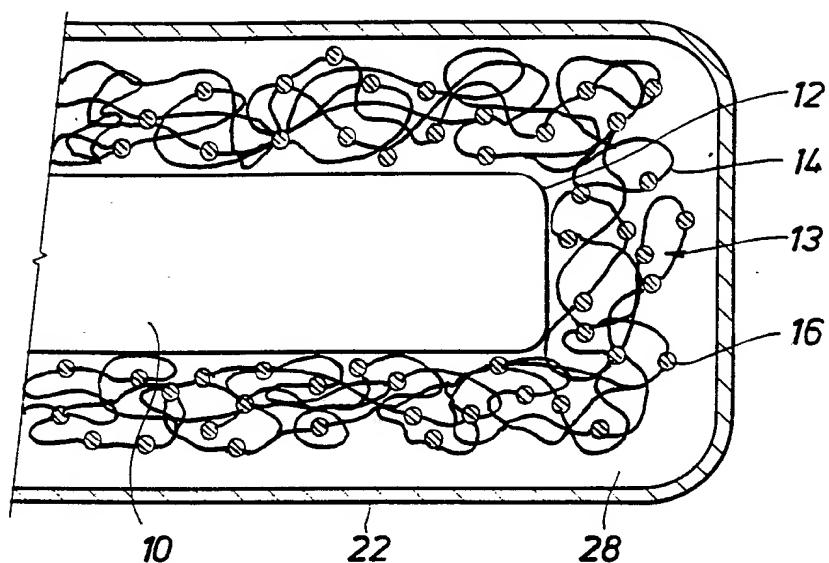


FIG. 6

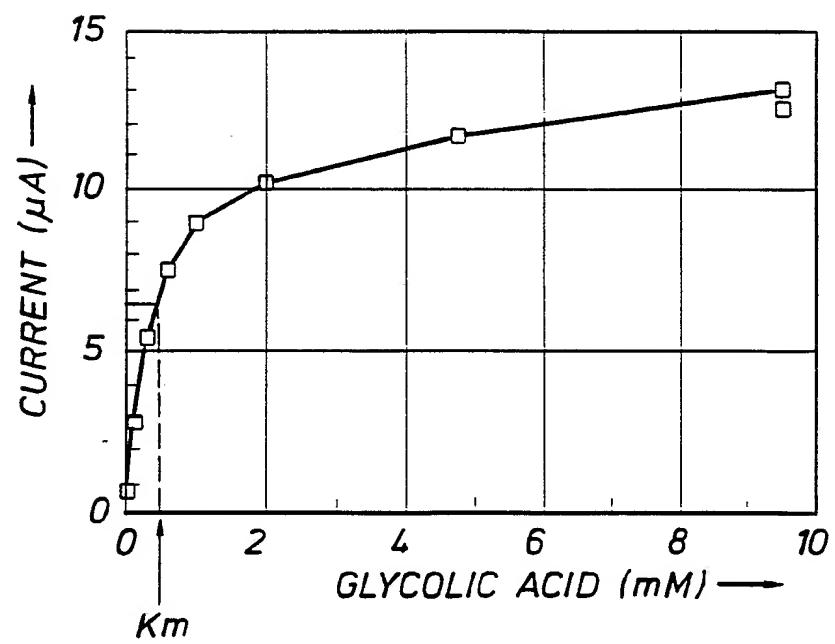


FIG. 7

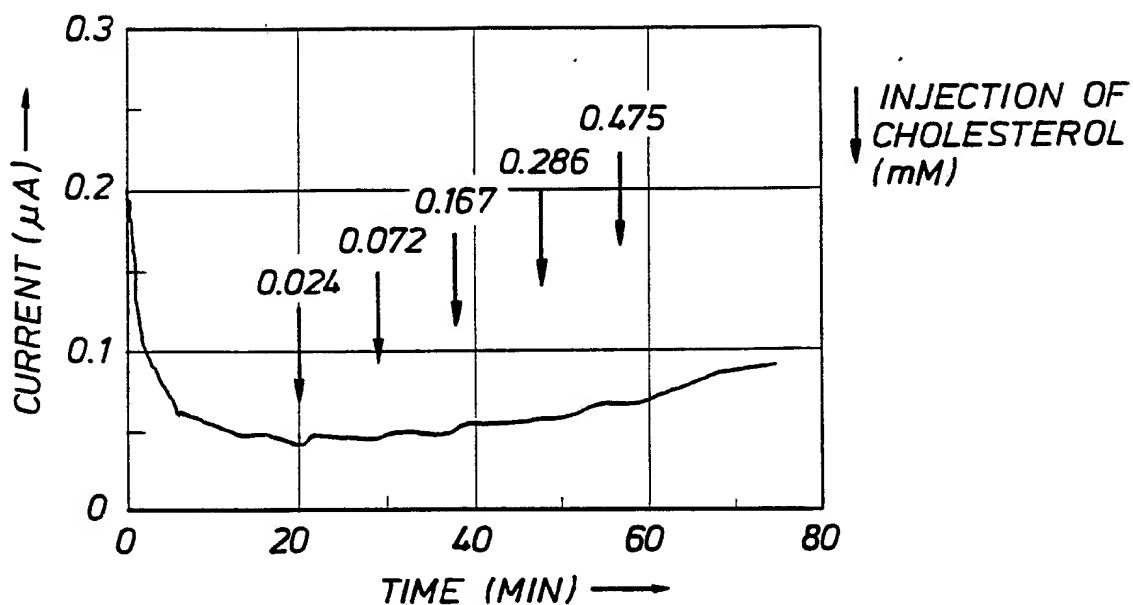


FIG. 8

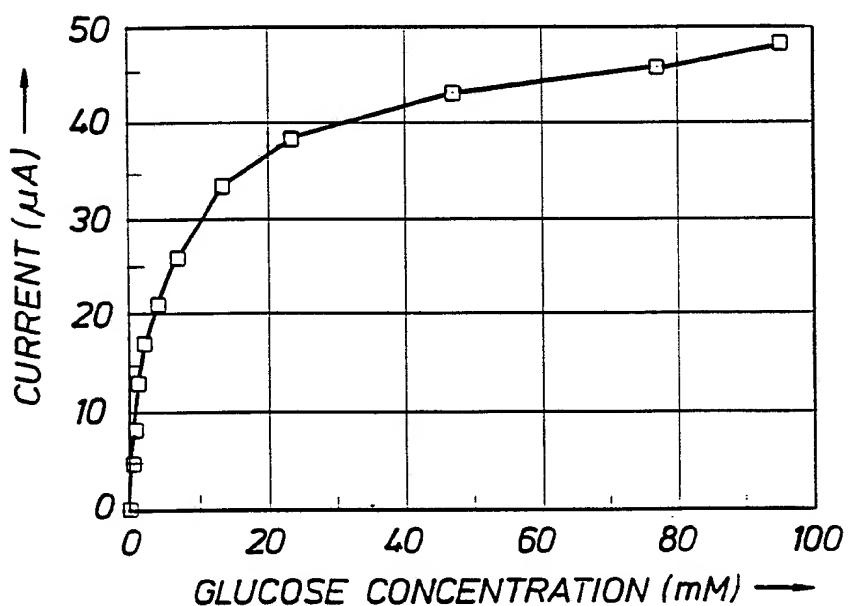


FIG.9

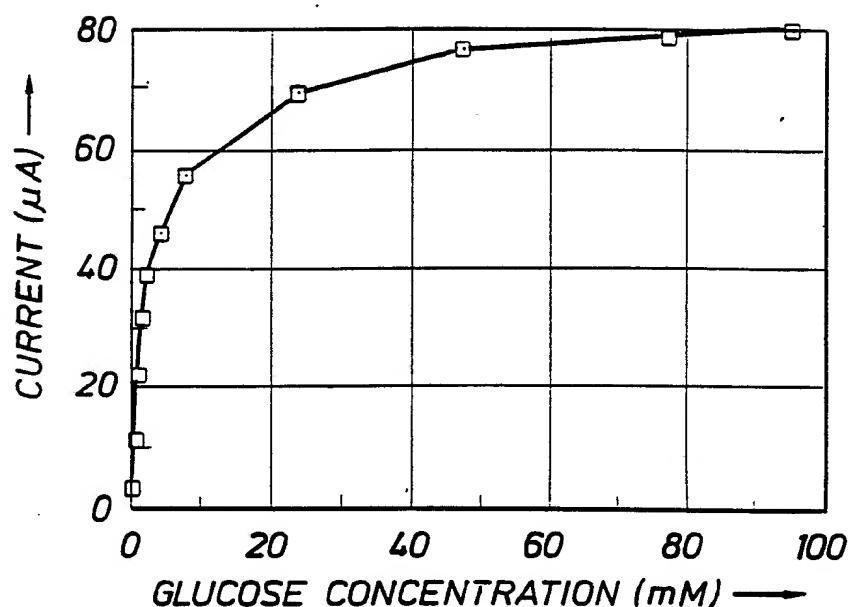
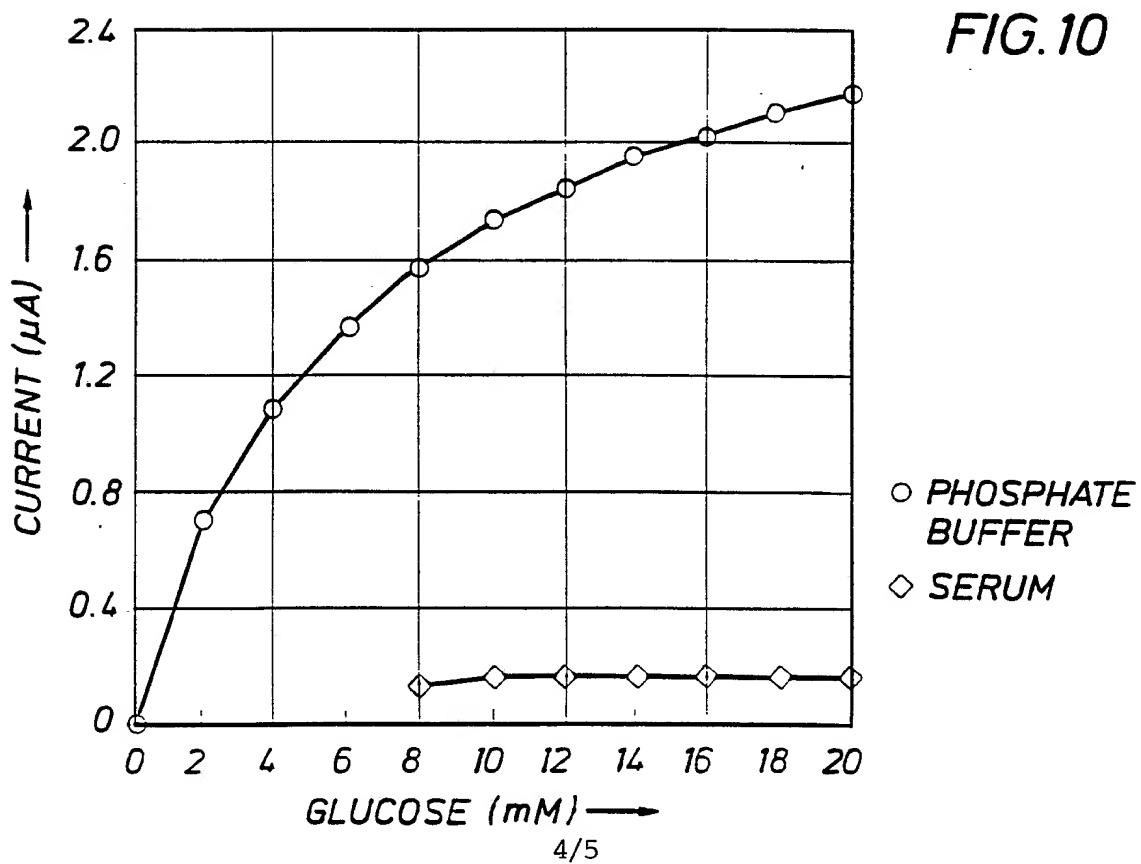


FIG.10



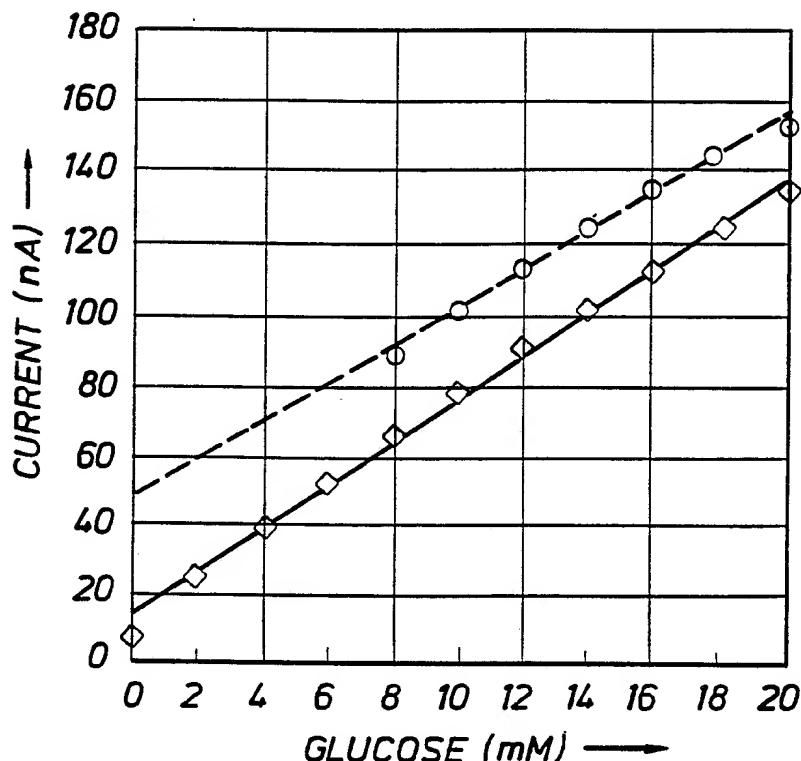


FIG. 11

○ PBS $0.08 \mu\text{A} / \text{mM cm}^2$
 ◇ SERUM $0.08 \mu\text{A} / \text{mM cm}^2$

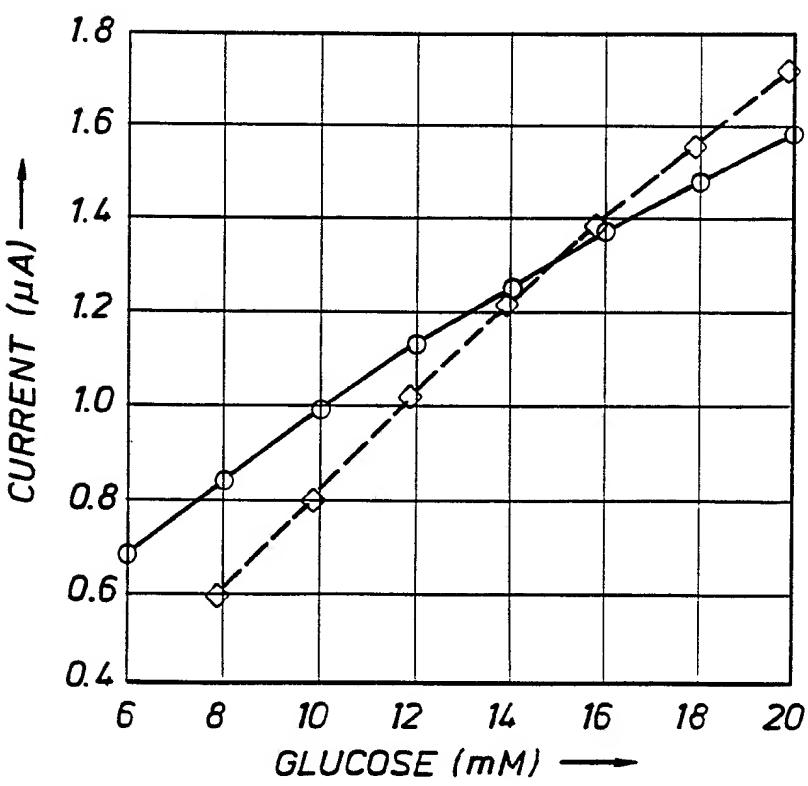


FIG. 12

○ PBS $0.91 \mu\text{A} / \text{mM cm}^2$
 ◇ SERUM $1.33 \mu\text{A} / \text{mM cm}^2$

INTERNATIONAL SEARCH REPORT

PCT/US93/02588

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 27/26
 US CL :204/403, 415, 153.12; 435/817, 288, 291

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 204/403, 415, 153.12; 435/817, 288, 291

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,927,516 (Yamaguchi et al) 22 May 1990.	1-16
A	US, A, 3,979,274 (Newman) 07 September 1976.	1-16
A	US, A, 4,545,382 (Higgins et al) 08 October 1985.	1-16

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

03 JUNE 1993

Date of mailing of the international search report

28 JUN 1993

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